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13. ABSTRACT ***(Maximum 200 Words)***

Apoptosis plays a critical role in growth and development of the mammary gland in normal and pathologic states. An important regulator of apoptosis is the bcl-2 oncogene, whose expression prevents apoptosis and is associated with poor responses to cancer therapies. Other bcl-2-related genes have been identified, defining a gene family with anti- and pro-apoptotic members. The molecular mechanisms which link bcl proteins to apoptosis are unclear. bcl-X_L forms ion channels in artificial membranes. To determine whether these proteins form or regulate ion channels in the endoplasmic reticulum in vivo, we have employed a novel Xenopus oocyte nuclear envelope patch-clamp technique. During the second funding period, we have developed a novel mammalian expression system for patch clamp electrophysiology of recombinant endoplasmic reticulum localized membrane proteins. The system has been validated, and it will now be possible to use it for expression of bcl-reated proteins. We discovered that caspase 3, a key intermediate in apoptosis pathways, cleaves the inositol trisphosphate receptor calcium channel in the endoplasmic reticulum, and causes it to become spontaneously activated, leaking calcium into the cytoplasm. This observation may provide a molecular insight into disruption of calcium homeostasis observed in apoptosis. We will determine whether other capsases have similar effects. In addition, we will examine the cellular consequqnces of this effect, and whether its modulation affects the time-course or extent of apoptosis.

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FOREWORD

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PI - Signature 7/30/07 Date

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INTRODUCTION

Apoptosis, the morphological and biochemical manifestation of programmed cell death, plays a critical role in maintaining homeostasis of tissue and organ cell number, and is involved in differentiation, growth and development (1-4). Mammary gland physiology is strongly influenced by apoptosis in both normal and pathologic states. Involution of the lactating gland is due to apoptosis of differentiated epithelial cells, and an emerging hypothesis is that dysfunction of the apoptotic pathways in mammary gland is significantly involved in the causes and progression of breast cancer (5-9). Thus, definition of the biochemical pathways involved in mammary gland apoptosis is an important goal in breast cancer research. An important regulator of apoptosis is the bcl-2 oncogene (2,5). Bcl-2 expression prevents apoptosis in several cell types and is associated with a poor prognosis in response to various cancer therapies in patients. Bcl-2 is normally expressed at high levels in some tissues, including mammary gland. More recently, other bcl-2-related genes have been identified, defining a gene family. Like bcl-2, some are anti-apoptotic, whereas others promote apoptosis. It is likely that the pro:anti -apoptotic expression level ratio regulates sensitivity to apoptosis. Breast cancer is associated with an altered ratio, which correlates with failure to respond to therapy and poor survival (7-9). Thus, many breast cancers may be diseases of apoptosis. The molecular mechanisms which link bcl proteins to apoptosis are undefined, although bcl proteins act at a critical juncture which integrates different death signals and activates a single death pathway. Intracellular [Ca^{2+}] and intracellular Ca^{2+} stores may be involved in regulating apoptosis, and expression of bcl-2 has been linked to alterations in Ca^{2+} signaling and in the handling of Ca^{2+} by intracellular stores, including the endoplasmic reticulum (ER) and mitochondria (4,10-13). The bcl proteins are localized to the outer mitochondrial membrane, ER membrane and outer membrane of the nuclear envelope. Recent studies suggest that bcl-related proteins are closely associated with permeability pathways in membranes. Cyotchrome c (CytC) release from mitochondria *in vitro* could be blocked by bcl-2. In addition, bcl-xL was demonstrated to form ion channels in artificial membranes. These data suggest that bcl proteins can form and/or regulate channels, perhaps for organic (e.g. CytC) and well as for inorganic (e.g. Ca^{2+}) molecules. Nevertheless, the physiological relevance of these data are questionable without measurements of channel activity in the membranes in which these proteins normally reside in cells. This has not been possible because the intracellular location of the membranes has prevented use of rigorous electrophysiological approaches, in particular the single-channel patch clamp technique. My laboratory recently developed novel technology for measuring ER- and nuclear envelope-localized ion channel activities (14,15). We proposed to employ this approach, together with recombinant bcl proteins, stably-expressing cell lines and expression systems, in a novel series of experiments designed to determine whether bcl-related proteins form ion channels in the ER and nuclear envelope, and whether these proteins regulate the activities and regulation of other permeability pathways which exist in these membranes. The specific aims are to: 1. Determine whether recombinant bcl-related proteins can form functional ion channels in the outer membrane of the nuclear envelope; 2. Determine whether expression of bcl-related proteins confers novel ion channel activities in the outer nuclear membrane; 3. Determine the role of bcl-like proteins in influencing the activities of resident ion channels in the nuclear envelope and the permeability of the nuclear pore. These studies may provide direct evidence for a biochemical function of proteins critically involved in apoptosis, mammary gland biology and breast cancer.

BODY

We proposed to undertake 3 specific aims during the 3 year granting period:

1. Determine whether recombinant bcl-related proteins can form functional ion channels in the outer membrane of the nuclear envelope.

2. Determine whether expression of bcl-related proteins confers novel ion channel activities in the outer nuclear membrane.

3. Determine the role of bcl-like proteins in influencing the activities of resident ion channels in the nuclear envelope and the permeability of the nuclear pore.

Our efforts during the first year had as their focus specific aim 2, with some attention also directed to specific aim 3. As proposed, we initiated experiments to determine whether heterologous expression of bcl-related proteins would result in novel ion channel activities in the outer membrane of the nuclear envelope. Our focus thus far has been on the *Xenopus* oocyte system, because of our familiarity with the procedures involved in the isolation of intact nuclei, patch clamp electrophysiology of the outer membrane, and expression of recombinant ion channels in this system. Because the *Xenopus* oocyte can express recombinant proteins, we reasoned that bcl-related proteins could be expressed and localized to the nuclear envelope, as in mammalian cells, and that patch clamp of the isolated nucleus could provide an opportunity to record ion channel activities which they might possess. Nevertheless, we did not detect novel channel activities, as we reported in our previous progress statement. We therefore refined our focus in two areas.

a. Development of a mammalian system for nuclear patch clamping. First, we considered that the oocyte expression system may not be optimal for the expression of recombinant mammalian proteins. We have therefore developed a comparable mammalian expression system that would enable patch clamp electrophysiology to be performed on isolated nuclei. To develop this system, we used Cos7 cells that had been transiently transfected with the rat type 1 inositol trisphosphate receptor. We have been able to routinely attain giga-ohn electrical seals on the nuclear membrane, validating the technical approach. Using transfected cells, we have been able to detect the activities of recombinant $InsP_3R$ channels, including wild-type and mutant constructs. Therefore, we have now established a mammalian cell system for proceeding with our work with the Bcl proteins. Two publications have resulted from this effort.

b. Second, we began to consider other molecular components of apoptosis pathways. Specifically, because intracellular [Ca^{2+}] and intracellular Ca^{2+} stores may be involved in regulating apoptosis, and the $InsP_3R$ has been recently shown to be a substrate of caspase 3 (16), we examined the effects of caspase 3 on $InsP_3R$ channel activity. Caspase 3 is a key executioner caspase involved in apoptosis pathways (17). The type 1 $InsP_3$ receptor contains one consensus site for cleavage by caspase 3, and it was recently shown to be a substrate for caspase 3 (16). We hypothesized that cleavage by caspase 3 of the $InsP_3R$ may link the apoptosis pathway to Ca^{2+} signalling. We examined the effects of purified recombinant caspase 3 on the ion channel properties of the *Xenopus* type 1 $InsP_3R$. Caspase 3 was included in the pipette solution. Control patches were performed with the pipette solution lacking the enzyme, or containing a specific caspase 3 inhibitor peptide. We found that caspase 3 activates the $InsP_3R$ channel. This effect does not require $InsP_3$, because inclusion of the $InsP_3$ competitive inhibitor heparin had no effect. The channels in the presence of heparin were not observed in control patches, or in patches with pipettes containing the capsase 3 inhibitor. This result represents the first observation of channel activity of the $InsP_3R$ that does not require $InsP_3$ ligand. It suggests that activation of caspase 3 during apoptosis could induce a spontaneous Ca^{2+} leak into the cytoplasm from the endoplasmic reticulum. We are continuing to work on this novel finding as part of a larger study that examines the roles of proteases in addition to the caspases.

Studies during the final year

Our studies during the final year have been largely influenced by novel results obtained by our collaborator Dr. Craig Thompson. In discussions with him, he indicated that his laboratory used gene expression micro-array analysis top discover genes whose expression was influenced by over-expression of bcl-X_L . They discovered that the major gene down-regulated by bcl-X_L expression was the type 1 $InsP_3R$ channel. This work was just published (18) They hypothesized that the $InsP_3R$ channel plays a central role in bcl-regulated apoptosis, possibly by regulating the extent to which calcium release from the endoplasmic reticulum influences mitochondrial function. Indeed, over-expression of the $InsP_3R$ inhibited the anti-apoptotic effect of bcl-X_L (18). These exciting

results have reinforced our ideas regarding the important role of the $InsP_3R$ in apoptosis. They suggest that factors that regulate the activity of the channel could be targeted for therapeutic purposes involving apoptosis and cell proliferation. We therefore set out to discover proteins that interact with the $InsP_3R$ that could possibly regulate its activity through protein-protein interactions. We undertook a yeast two-hybrid screen to discover proteins that interact with the $InsP_3R$. Using the first 600 residues of the rat type 3 channel, a region that contains the $InsP_3$-binding domain, we screened a human brain cDNA library and identified a previously described gene family termed CaBP (19,20). CaBPs, originally cloned from retina (21), belong to the neuronal Ca^{2+}-binding protein (NCBP) subset of EF-hand-containing proteins. NCBP family members include recoverin, hippocalcin, neuronal calcium sensor-1 (frequenin), visinin, VILIPs, GCAPs and KChips (calsenilin) (20-24). NCBPs are similar to calmodulin (CaM) family members in having 4 Ca^{2+}-binding EF hands motifs, but they are distinguished in that one or two of the motifs may be non-functional in Ca^{2+} binding, and they frequently are myristoylated at the NH_2-terminus (21,22). The CaBP sub-family is distinguished by its unique combination of functional EF-hand motifs, with the second EF hand disabled, and by the presence of an extra turn in the central alpha helix. Five members have been identified. Alternative splicing of the N-terminus also generates long and short forms of CaBP1 and CaBP2. Another protein termed caldendrin is a third splice variant of CaBP1 (21,25-27). A protein containing the distal two EF hands has been termed calbrain (28), but it is probably a partial clone of CaBP1 (21). Our screen identified caldendrin and CaBP1, which share identical C-termini containing the EF-hand motifs. The longest clone encompassed the C-terminal 256 aa containing all 4 EF hands, whereas the shortest represented the terminal 103 aa containing 3 EF hands.

To confirm the interaction in mammalian cells, a GFP-tagged short variant of human CaBP1 (s-CaBP1-GFP) (Fig. 1B) was generated and expressed in Cos-7 cells. Immunoprecipitation (IP) of $InsP_3R$-3 efficiently co-IPed CaBP1, detected using a CaBP1 antibody provided by our collaborator Dr. F. Haeseleer (Fig. 1B, lane 3). In the reciprocal experiment, IP of CaBP1 co-precipitated $InsP_3R$-3 (Fig. 1B, lane 1). To determine if the N-terminal 600 aa represented the only region involved in binding to CaBP1, *in vitro* "pull-down" assays were employed. Full-length r-$InsP_3R$-3 or a mutant $InsP_3R$-3 lacking the first 600 aa (Δ1-600-$InsP_3R$-3) were expressed in *Xenopus* oocytes (Fig. 1C, lanes 3 and 1, respectively). Full-length $InsP_3R$-3 was efficiently pulled down by GST-c-CaBP1 (Fig. 1C, lane 3), whereas Δ1-600-$InsP_3R$-3 was not (Fig. 1C, lane 2). Therefore, the N-terminal 600 aa appear to be both necessary and sufficient for interaction with CaBP1.

All three channel isoforms bound to CaBP1 (Fig. 1D). Because transiently-expressed recombinant $InsP_3Rs$ do not form hetero-oligomers with endogenous $InsP_3Rs$ in Cos-7 cells, we transfected Cos-7 cells with r-$InsP_3R$-1 and -3 and determined that they efficiently bound to CaBP1 (Fig. 1E), demonstrating that CaBP1 can interact with homo-tetrameric types 1 and 3 $InsP_3Rs$. Similar experiments weren't performed with the type 2 isoform, but its high sequence homology with the other two suggests that it too likely binds directly to CaBP1.

Treatment of cells with the Ca^{2+} ionophore ionomycin (2 μM) enhanced the amount of s-CaBP1-GFP detected in $InsP_3R$-3 immunoprecipitates (Fig.2A), suggesting that Ca^{2+} enhances the interaction. Divalent cation dependencies of binding were investigated by fixing [Ca^{2+}] and [Mg^{2+}] in lysates. In 0-Ca^{2+}, Mg^{2+} had little effect on binding (Fig.2B, lane 1 vs. 2) whereas raising [Ca^{2+}] to 500 μM enhanced binding of CaBP1 to $InsP_3R$ (Fig.2B, lane 3) by >20-fold compared with that observed in 0-Ca^{2+}, although binding was observed in absence (~ 2-5 nM) of Ca^{2+} (Fig.2B, lane 2). Mutations to ala of 6 conserved residues involved in Ca^{2+} coordination in functional EF hands eliminated binding (Fig. 2C). Wild-type CaBP1 binding to the $InsP_3R$ was strongly enhanced when [Ca^{2+}] in the lysates was raised from 100 nM to ~ 5 μM (Fig. 2D), with Ca^{2+} affinity of ~ 1 μM (Fig. 2E). Ca^{2+}-induced CaBP1 binding to the $InsP_3R$ therefore occurs over a physiologically-relevant range of $[Ca^{2+}]_i$, suggesting that *in vivo* changes in $[Ca^{2+}]_i$ may regulate the interaction between the two proteins.

CaBPs are protein ligands of the $InsP_3R$ channel. The functional consequences of the interaction of CaBP1 with the $InsP_3R$ were examined by patch clamping the *X*-$InsP_3R$-1. We thought that CaBP would inhibit gating. Purified s-CaBP1 (1 μM) together with $InsP_3$ (33 nM) were included in the pipette solution at optimal [Ca^{2+}]. However, robust channel activity was similarly observed in the presence or absence of CaBP1 (Fig. 3B, C). We then examined CaBP1 in the absence of $InsP_3$. Surprisingly, channel gating with high P_o (~0.8) was observed when 1 μM s-CaBP1 was included in the pipette solution (Fig. 3D, 15/17 patches). Activation was caused by CaBP1, because patches lacking CaBP1 did not display channel activities (Fig. 3E, 33/36 patches). The triple-EF-hand mutant protein (1 μM) failed to activate the channel (Fig. 3F, 10/11 patches). Thus, Ca^{2+}-dependent binding of CaBP1 to the $InsP_3R$ mediates channel activation. Activation of channels with high P_o was observed with CaBP1 reduced to 10 nM (Fig. 3H); thus CaBP1 is a high affinity activator of the $InsP_3R$.

Our functional and biochemical results demonstrate that CaBP1 is a high-affinity, specific protein ligand of the $InsP_3R$, the Ca^{2+}-dependent binding of which activates gating in the absence of $InsP_3$ with features (P_o, gating kinetics) remarkably similar to those activated by $InsP_3$.

Purified bovine s-CaBP2 and mouse CaBP5 (1 μM each), with C-terminal sequences ~85 % similar to human CABP1/caldendrin, each stimulated gating with high P_o (Fig. 3 I,J). These results therefore identify the CaBP Ca^{2+}sensors as a family of protein ligands of the $InsP_3R$ channel.

CaBP1/caldendrin as well as other NCBPs belong to a super-family of EF-hand containing proteins, of which CaM is the prototype. CaM has been implicated in regulation of the $InsP_3R$ and it may affect $InsP_3$ binding, possibly by interacting within the $InsP_3$ binding region. To determine if CaM could bind to the CaBP1-interacting region, we performed *in vitro* competition experiments. Purified proteins were added to cell lysates, from which $InsP_3R$-3 was pulled down by GST-c-CaBP1. s-CaBP1 competitively inhibited binding of $InsP_3R$-3 with apparent affinity of ~ 25 nM (Fig. 2 F,G). In contrast, CaM, even at 5 μM, had little effect (Fig. 2H), indicating that it does not interact well with the CaBP-binding site. Furthermore, CaM (12 μM) never activated channel gating (Fig. 3K, 17/17 patches) in membrane regions where c-CaBP1 did (Fig. 3L, 13/14 patches), in accord with the binding data. Thus, interactions of CaBPs with the $InsP_3R$ are highly specific ones that are not recapitulated by CaM.

CaBP1 and $InsP_3R$ interact and co-localize in brain. CaBP1 and caldendrin are expressed in specific cell types in retina and throughout the brain, including cortex, cerebellum and hippocampus, whereas CaBP2 and 3 be retina-specific (20,25-27). Expression in brain appears to localized to neuronal somato-dendritic compartments, especially at dendritic post-synaptic densities (25-27). $InsP_3R$ is widely distributed throughout the brain with $InsP_3R$-1 most highly expressed, and it is also localized in neuronal somatic and dendritic compartments (29-31).

To verify interaction of endogenous $InsP_3R$ and CaBP1, we performed IP and co-localization experiments. CaBP1 was present in IPs of $InsP_3R$-1 or $InsP_3R$-3 from whole rat brain (Fig. 4A). IP of $InsP_3R$-1 from cerebellum, where it is expressed at very high levels in Purkinje cells (32,33) co-IPed CaBP1. Like $InsP_3R$-1, staining for CaBP1 in cerebellum was strong in Purkinje cell somas and in their dendrites (Fig. 4B) with extensive co-localization (Fig. 4B-F) that was nearly complete within striated structures (Fig. 4C-F) that are likely smooth ER that runs immediately under the plasma membrane (subsurface or hypolemmal cisternae; 32,34). Thus, CaBP1 is membrane-localized, perhaps via its myristoylated N-terminus, close to the $InsP_3R$ in neurons.

KEY RESEARCH ACCOMPLISHMENTS during the entire granting period.

- Development of a mammalian cell system for expression of ER-localized recombinant proteins and patch clamp electrophysiology of their isolated nuclei.

- Identification of an effect of caspase-3 mediated cleavage of the $InsP_3R$ on its ion channel activity, inducing spontaneous Ca^{2+} channel activity.
- Identification of a family of protein ligands of the $InsP_3R$.

REPORTABLE OUTCOMES

Boehning, D., S.K. Joseph, D.-O.D. Mak and J.K. Foskett. 2001. Single-channel recordings of recombinant inositol trisphosphate receptors in mammalian nuclear envelope. Biophysical J. **81**:117-124.

Boehning , D., D.-O.D. Mak, J.K. Foskett and S.K. Joseph. 2001. Molecular determinants of permeation and selectivity in inositol 1,4,5-trisphosphate receptor Ca^{2+} channels. J. Biol. Chem. **276**:13509-13512.

Yang, J., S. McBride, D.-O. D. Mak, F. Haeseleer, K. Palczewski and J. K. Foskett. 2002. Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor Ca^{2+} release channels. Proc. Nat. Acad. Sci. **99**:7711-7716.

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FIGURE LEGENDS

Figure 1. Interaction of the $InsP_3R$ with CaBP1. ***A***. Domain structures of CaBPs and calmodulin. ***B*** . Co-IP of CaBP1 and $InsP_3R$-3 from control COS-7 cells (lanes 2 and 4) and COS-7 cells transiently transfected with s-CaBP1-GFP (lanes 1 and 3). Immunoprecipitates were probed with an $InsP_3R$ type 3-specific antibody (top) or anti-CaBP1 antibody (bottom). COS-7 cells do not express endogenous CaBP1 (lane 4). ***C***. *In vitro* binding of $InsP_3R$-3 to CaBP1 requires the NH_2-terminal 600 residues of the $InsP_3R$. Lysates from *Xenopus* oocytes expressing full-length r-$InsP_3R$-3 (lane 3, lysate from 50 oocytes) or type 3 $InsP_3R$ lacking the first 600 residues (Δ1-600-$InsP_3R$-3) (lane 2, lysate from 50 oocytes) were incubated with GST-CaBP1, and bound $InsP_3R$ was detected with a COOH-terminal $InsP_3R$-3 antibody. Expression of Δ1-600-$InsP_3R$-3 was verified by IP and Western blotting (lane 1, lysate from 14 oocytes). ***D***. All three mammalian $InsP_3R$ isoforms interact with CaBP1 *in vitro*. COS-7 cell lysates were incubated with GST-c-CaBP1, and bound $InsP_3R$ was detected with isoform-specific antibodies. Type 1 was pulled down with GST only (in 5 mg lysate, lane 1) or with GST-c-CaBP1 (from 5 mg lysate, lane 2,); Type 2 in GST-c-CaBP1 pull-down from 1.25 mg lysate (lane 3); Type 3 present in 50 μg lysate (lane 4), in pull-down with GST only (from 1.25 mg lysate), and in pull-down with GST-c-CaBP1 (from 1.25 mg lysate). Equivalent GST-fusion protein concentrations were present in *in-vitro* binding reactions (right panel, Western blot with anti-GST antibody). Intensities are within the linear range. Inspection of intensities and normalization of lysates used indicates stoichiometric interaction of $InsP_3R$ and CaBP1. ***E***. Homotetrameric rat types 1 and 3 $InsP_3R$ isoforms interact with CaBP1. Lysates from control COS-7 cells (-) or COS-7 cells transfected with types 3 (3, left panel) or 1 (1, right panel) $InsP_3R$ were incubated with GST-CaBP1, and bound $InsP_3R$ was detected with isoform-specific antibodies. Type-3 (left panel): 5 μg or 250 μg cell lysate used in first and second pairs of lanes, respectively. Type 1 (right panel): 25 μg or 250 μg cell lysate used in third and fourth pairs of lanes, respectively. Because of high level over-expression of the $InsP_3R$, pull-down intensity is not proportional to the amount of $InsP_3R$ input in this experiment.

Figure 2. Ca^{2+} dependence of CaBP1-$InsP_3R$ interaction. ***A***. Elevation of $[Ca^{2+}]_i$ enhances the interaction of the $InsP_3R$ with CaBP1. Co-IP, using type 3 $InsP_3R$ antibody, of CaBP1 with $InsP_3R$-3 from lysates of CaBP1-GFP-transfected COS-7 cells (left panel) exposed (+) or not (-) for 2 min to the Ca^{2+} ionophore ionomycin (2 μM). Immunoprecipitates (left) or cell lysates (right; 5 μg each) were probed for $InsP_3R$-3 (upper) or CaBP1 (lower). Ionomycin enhanced the amount of CaBP1 detected in immunoprecipitates, which contained equal amounts of $InsP_3R$ (lanes 1 and 2, top) and s-CaBP1-GFP (lanes 3 and 4, bottom). ***B***. *In vitro* binding of $InsP_3R$-3 to CaBP1 is specifically enhanced by Ca^{2+}. COS-7 cell lysates, with free $[Mg^{2+}]$ and $[Ca^{2+}]$ fixed to 500 μM Mg^{2+}/0 Ca^{2+} (left lane), 0 Mg^{2+}/0 Ca^{2+} (middle lane) or 0 Mg^{2+}/500 μM Ca^{2+} (right lane) were incubated with GST-c-CaBP1, and bound $InsP_3R$ was detected with type-3-specific antibody. ***C***. Functional Ca^{2+}-binding EF-hands are required for CaBP1 to interact with the $InsP_3R$. Endogenous $InsP_3R$-3 from COS-7 cell lysate was pulled down with GST-CaBP1 (wt) but not with GST-CaBP1 triple-EF-hand mutant (mut). Equivalent GST-fusion protein concentrations were present in *in vitro* binding reactions (right panel, Coomassie stain). ***D***. $[Ca^{2+}]$ dependence of *in vitro* interaction of CaBP1 and $InsP_3R$. Endogenous $InsP_3R$-3 in COS-7 cell lysate (1.25 mg) with $[Ca^{2+}]$ fixed as indicated was pulled down with GST-c-CaBP1 and probed with $InsP_3R$-3 antibody. ***E***. $[Ca^{2+}]$-dependence of $InsP_3R$-3 interaction with CaBP1 by quantitative densitometry of gels similar to that shown in (D) (n =3) with data normalized to binding observed in 500 μM Ca^{2+}. ***F***. CaBP1 binding affinity for the $InsP_3R$-3. Endogenous $InsP_3R$-3 was pulled down with GST-CaBP1 from COS-7 cell lysates (1.25 mg) containing defined concentrations of s-CaBP1. ***G***. Quantitative analysis of competition for CaBP1 binding to $InsP_3R$-3 by s-CaBP1 with data normalized to binding in the absence of added s-CaBP1. ***H***. Specificity of the interaction with the $InsP_3R$ of CaBP1 vs calmodulin (CaM). Endogenous COS-7 cell $InsP_3R$-3 was pulled down with GST-c-CaBP1 from lysates (1.25 mg) supplemented with various concentrations of CaM or s-CaBP1.

Figure 3. Typical patch-clamp current records from outer membrane patches obtained from isolated *Xenopus* oocyte nuclei. Applied potential = 20 mV. The arrows indicate the closed channel current level. The pipette solutions contained: agonists as indicated. Current traces D and E, F and G, and K and L (those enclosed with braces) were recorded with membrane patches obtained from the same region of the same oocyte nuclei. Free Ca^{2+} concentrations used in all pipette solutions were optimal for achieving maximum channel P_o (1.5 – 21 μM ; (13) .

Figure 4. Interaction of $InsP_3R$ and CaBP1 in brain. ***A***. Co-IP of CaBP1 with $InsP_3R$-1 (left and right) or $InsP_3R$-3 (left) from whole rat brain (left) and cerebellum (right), but not from non-neural tissues (left). Immunoprecipitates probed with anti-CaBP1 antibody. Reciprocal experiment could not be performed because the CaBP antibody is directed against the same region to with the $InsP_3R$ binds. ***B-F***. Confocal immunolocalization of $InsP_3R$-1 and CaBP1 in rat cerebellum sagital sections. ***B***. Low magnification. CaBP1 (green) and $InsP_3R$-1 (red) are localized to Purkinje cell somas (PC) and their dendrites in the molecular layer (Mol) (co-localization indicated by yellow). CaBP1 (but not $InsP_3R$-1) is also localized to unidentified fine structures within the granular cell layer (Gr) and to stellate cells (arrow) in the molecular layer. ***C-F***. Higher magnification demonstrating subcellular co-localization on endoplasmic reticulum. ***C***. Striated co-localization (yellow) in Purkinje cell primary dendrite. ***D-F***. Dendritic tips of Purkinje cells. CaBP1 (**E**; green) and $InsP_3R$-1 (**D**; red) are co-localized (**F**; yellow) to linear sub-regions within thin dendrites (arrowheads).

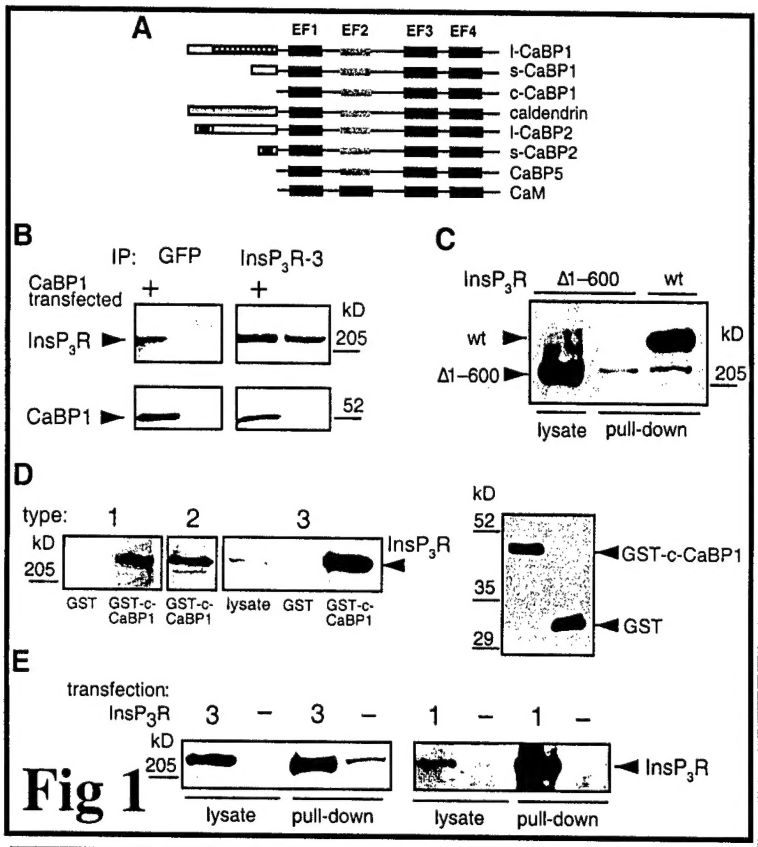


Fig 1

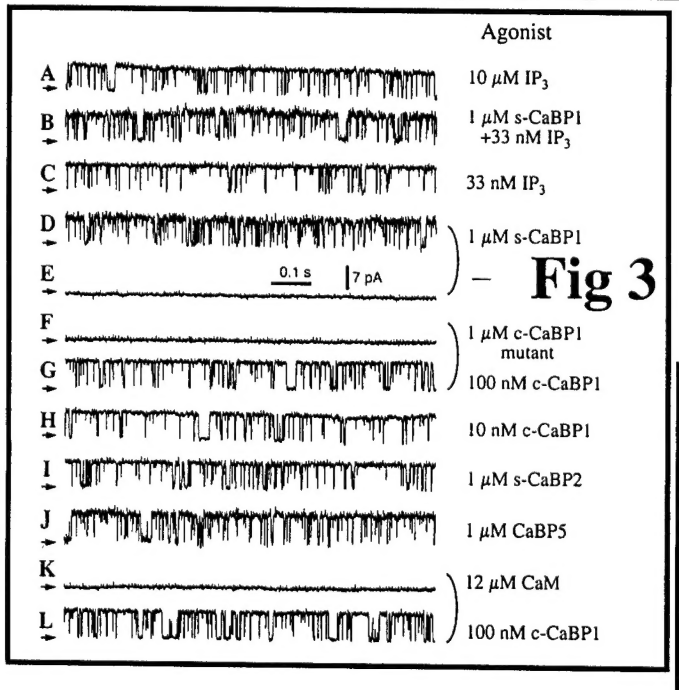


Fig 3

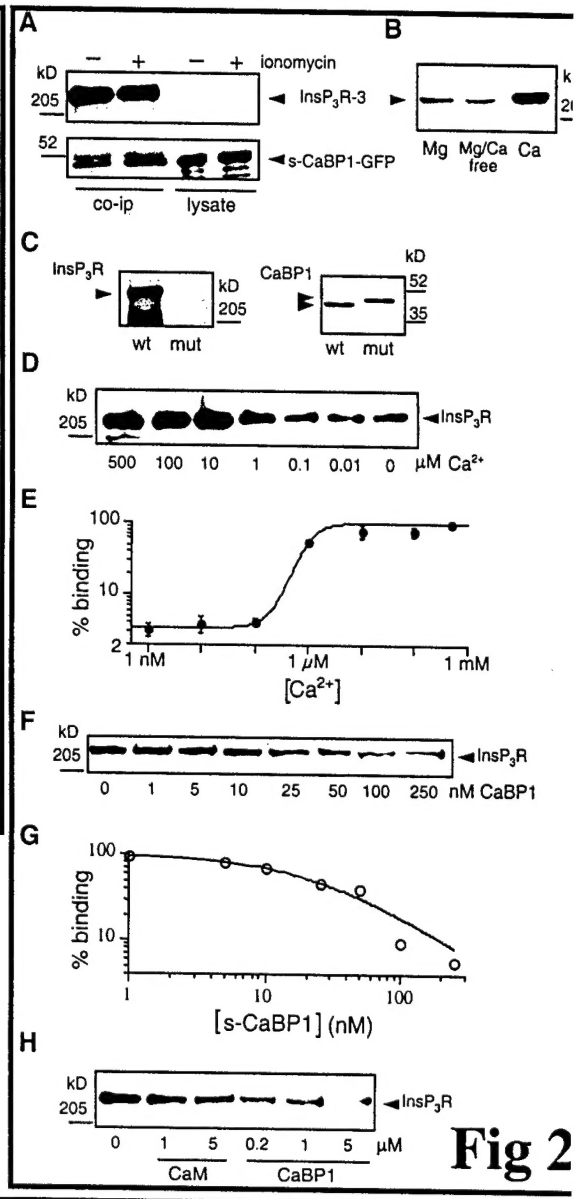
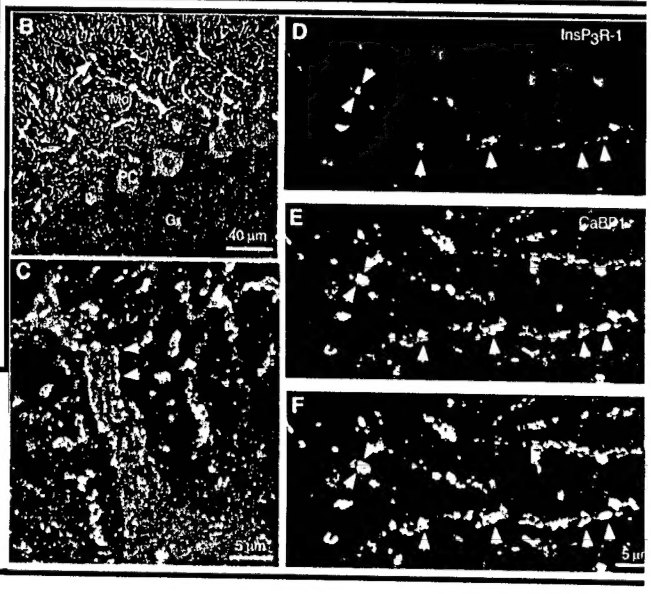


Fig 2



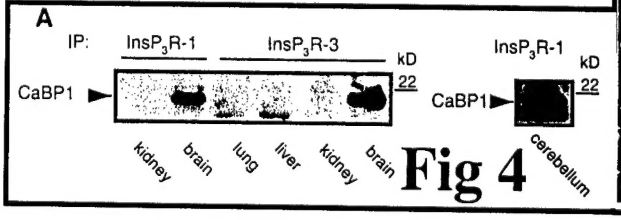


Fig 4